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Proteomics of *Colwellia psychrerythraea* at subzero temperatures - a life with limited movement, flexible membranes and vital DNA repair

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Summary

Cold adaptation in high latitude marine bacteria demonstrates that life persists in extreme environments. The mechanisms that allow psychrophilic bacteria to remain metabolically active at subzero temperatures are due to the form and function of their proteins. We present the first proteomic evidence of physiological changes of the marine psychrophile *Colwellia psychrerythraea* 34H (*Cp*34H) after exposure to subzero temperatures (-1, -5, and -10°C) through 8 weeks. *Cp*34H [3H]-leucine and [3H]–thymidine incubations reflected active protein and DNA turnover through time with highest measurements observed at -1°C and lowest at -10°C. Mass spectrometry-based proteomics identified 1763 proteins across four experimental treatments, each with three biological and two technical replicates. Protein abundance was compared between the different treatments to understand effects of the independent variables of temperature and time and, more importantly, the comprehensive proteomic shifts that occurred between the subzero treatments after acclimation, emphasizing the physiological mechanisms employed during transition to and incubation in ice. Protein groups revealed to be differentially abundant included DNA repair, flagellum assembly, iron and nitrogen metabolism, membrane flexibility, and protein synthesis and folding. Proteomic evidence indicated that multiple flagellar proteins were reduced after 8 weeks in ice, confirming that metabolic energy for motility decreased. Increases in DNA repair proteins combined with low overall DNA synthesis at -10°C suggests that DNA repair also becomes a vital strategy during ice transition. This study provides novel insight into the extensive molecular changes that occur in cold-adapted marine organisms to sustain cellular function in their habitat.

Introduction

The mechanisms that enable bacteria to be metabolically active in ice at subzero temperatures have been of considerable interest to studies of polar marine and terrestrial ecology, cryobiology, astrobiology, climatology and atmospheric sciences (Junge et al., 1998; Junge et al., 2004a; Christner et al., 2008b; Christner et al., 2008a; Deming, 2009; Dieser et al., 2014; Tuorto et al., 2014). Despite several studies into the mechanisms of subzero microbial activity in terrestrial habitats (e.g., Mykytczuk et al., 2013; Dieser et al., 2014) the molecular nature of these in-ice processes has yet to be explored for marine bacteria.

The microbial composition and metabolic activity of polar sea ice bacteria has been typically described using melted sea-ice samples (for reviews see :Deming, 2010; Junge et al., 2011). It is challenging to make *in vivo* evaluations of the nature of adaptations (including molecular) that organisms adopt to facilitate activity and survival in ice, due to the difficulty of recreating complex environmental conditions, such as sea ice, within the laboratory. Despite experimental difficulties, research into the elusive nature of microbial processes at subzero temperatures remains active (e.g., Bakermans

and Skidmore, 2011; Piette et al., 2011; Collins and Deming, 2013; Dieser et al., 2013; Tuorto et al., 2014). The emerging picture of microbial metabolism at temperatures below -5°C suggests that low rates of metabolism in psychrotolerant organisms may offset cellular and macromolecular damage, thereby allowing for survival over prolonged time frames (Amato and Christner, 2009; Amato et al., 2009, Dieser et al, 2014). In contrast, the subzero molecular microbial activities of true psychrophiles remains to be reported. Here we present observations of differences in the proteomic compositions of the marine psychrophile and halophile *Colwellia psychrerythraea* strain 34H (*Cp*34H) cultures, which were incubated at different temperatures relevant to the polar environment (-1, -5, and -10°C) to suggest mechanisms of cold adaptation over long periods (8 weeks). The genome of *Cp*34H contains evidence of the potential for maintenance of membrane fluidity, the synthesis of extracellular polysaccharides and enzymes, and the uptake and synthesis of compounds conferring cryotolerance (Methe et al., 2005; Collins and Deming, 2013). More recent genomic analyses on permafrost bacteria revealed evidence for similar molecular changes, suggesting these adaptation strategies are key to survival in a variety of ice-covered environments (e.g., Bergholz et al., 2009; Ayala-del-Rio et al., 2010; Mykytczuk et al., 2013).

The genomics era has produced thousands of complete microbial genome sequences, allowing for a more thorough examination of genetic diversity and complexity than has previously been possible (DeLong and Karl, 2005). However, making connections between cellular function and environmental ecology based on genomic information alone remains challenging (Bertone et al., 2004; Stolc et al., 2005; Birney et al., 2007), whereas the implementation of proteomics provides a valuable tool to more robustly investigate these links (Pandey and Mann, 2000; Renuse et al., 2011). At subzero temperatures multiple strategies are required to overcome the temperature-dependent diffusive and enzymatic kinetics for carbon and nitrogen uptake and integration (Feller, 2003; Methe et al., 2005). To date, multiple labs that have examined protein abundance by cold adapted archaea and bacteria in liquid culture and have found significant shifts in protein abundance as temperature decreases (Saunders et al., 2006; Bakermans et al., 2007; Kawamoto et al., 2007; Casanueva et al., 2010; Williams et al., 2010; Campanaro et al., 2011; Piette et al., 2011; Williams et al., 2011; Jagannadham and Chowdhury, 2012; Pereira-Medrano et al., 2012; Piette et al., 2012). The study presented here provides the first proteomic investigation of a marine psychrophile and the metabolic shift that this species of marine bacterium undergoes when frozen into saline ice and exposed to sustained subzero temperatures, mimicking what occurs in polar regions over the winter months.

We report the results of experiments monitoring *Colwellia psychrerythraea* strain 34H subzero proteomics and metabolic activity. In order to identify the molecular strategies that enable *Cp*34H cells to maintain cellular integrity and activity at subzero temperatures in a saline ice environment, we examined and compared whole-cell

proteomic profiles of *Cp*34H after 24 hours at -1°C and 8 weeks at -10°C. The presented findings communicate critical biochemical strategies adopted by a marine psychrophile allowing it to remain metabolically active within saline, subzero environments. These results provide insight into the interaction between an Arctic bacterium and its environment, an ecologically important relationship given that 20% of the Earth's surface is frozen as permafrost, glacial ice, polar sea ice, and snow cover (Deming and Eicken, 2007).

Results

DNA and protein metabolism

[3H]-leucine (leu) and [3H]-thymidine (thy) incorporation assays were performed in parallel over 8 weeks to evaluate long term metabolic activity and growth at subzero temperatures. Subzero incubation (-1, -5, and -10°C) of *Cp*34H revealed [3H]-leu incorporation was highest in the cells incubated at -1°C across all time points (6.1E5 $\pm$ 3E4 dpms after 24 hours, 5.1E5 $\pm$ 2E4 dpms after 8 weeks) and lowest for cells incubated at -10°C (1.1E5 $\pm$ 6E4 dpms after 24 hours, 1.5E5 $\pm$ 8E4 dpms after 8 weeks) (Figure 1A, Table S1). [3H]-thy incorporation assays performed in parallel revealed highest [3H]-thy incorporation in cells incubated at -1°C (3.2E4 $\pm$1.5E3 dpms after 24 hours, 3.4E4 $\pm$ 1.0E3 dpms after 8 weeks; Figure 1B, Table S1) and the lowest at -10°C (3.95E3 $\pm$ 2.1E2 dpms after 24 hours and 3.48E3 $\pm$ 9E1).

High bacterial growth rates (determined based on [3H]-leu incorporation data) over the first 24 hours of each incubation occurred at all temperatures: 8.6E-2 $\pm$ 3.1E-3 gC-Leucine [gC bacterial biomass]$^{-1}$ hr^{-1} and 2.31E-2 $\pm$ 5.1E-3 gC-Leucine [gC bacterial biomass]$^{-1}$ hr^{-1} at -1 and -10°C, respectively.

Previous studies investigating metabolic activities of *Cp*34H at subzero temperatures have flash frozen samples in liquid nitrogen (LN_2) prior to incubation at the desired subzero temperature (Junge et al., 2006). Examinations into the effects of flash freezing indicated significant differences in incorporation of [3H]-leu between those bacteria that were flash frozen and those immediately placed at their incubation temperature. As a result, in this study flash freezing was not used so that cell viability was not impacted (Text S1, Figure S1)

Proteomics

A total of 1763 *Colwellia psychrerythraea* proteins were identified from the combined results (Dataset S1). Total spectral counts of all combined experiments revealed translation elongation factor Tu to be the most abundant enzyme identified at these cold temperatures (Table 1). In addition, alanine dehydrogenase, an oxidoreductase involved in the generation of α-keto acids, and several other enzymes involved in transcription and protein synthesis were identified at high copy numbers. A similar number of proteins was identified in each bioreplicate/treatment group (1519-1586), indicating that proteins were successfully maintained even at -10°C after 8 weeks and intracellular

proteolytic degradation was not enhanced or accelerated.

Proteins were identified across 1304 different Gene Ontology categories (GO identifiers). Of note were proteins involved in the processes of temperature shock - heat and cold - (chaperone protein DnaJ, RNA polymerase sigma factor RpoH, chaperone protein DnaK, cold-shock DNA-binding domain family protein), osmolyte regulation (sarcosine oxidase, betaine aldehyde dehydrogenase, choline/carnitine/betaine transport family), and polymer secretion (biopolymer transport exbB and exbD proteins). Constitutive presence of these cold adaptation proteins across the treatments suggests that they are required by *Cp*34H for metabolic success below 0^{o}C.

NMDS of biological replicates revealed the clustering of samples by temporal and temperature conditions (Figure 3). NMDS analysis of the total spectral counts of identified proteins from the 11 individual samples (biotriplicate samples from -1°C 24 hours, -10°C 24 hours, and -10°C 8 weeks, bioduplicate from T0) grouped into 3 distinct populations corresponding to 1) T0 and -10°C 24 hours, 2) -1°C 24 hours, and 3) -10°C 8 weeks (Figure 3). The spatial proximity of T0 and -10°C 24 hours indicates that there was little change to the *Cp*34H proteome after 24 hours at -10°C, potentially a sign that cells did not undergo many changes to protein abundances (i.e. did not yet change abundance of ice- and salt-adapted proteins within the first 24 hours of freezing). This is especially striking given the larger proteomic changes that occurred after 24 hours at -1°C. ANOSIM revealed a significant difference in proteomic profile based on temperature and incubation time ($R = 0.0244$, $p = 0.001$).

We evaluated the effects of time and temperature as independent variables and we examined the proteomes of *Cp*34H at -1^{o}C after 24 hours to *Cp*34H at -10^{o}C for 8 weeks to determine which proteins drive the differences in success after exposure to these conditions. Comparing the expressed proteome from 24 hours of incubation at -1°C to the expressed proteome of *Cp*34H after 8 weeks at -10^{o}C highlights specific adjustments required when *Cp*34H has sufficient time to acclimate to the transition to ice and adjust to the slower kinetics of life at -10^{o}C. These extensive environmental and physiological changes are reflected in the NMDS analysis, where these two treatment groups show the greatest dissimilarity in proteomic profiles. Although the NMDS demonstrates that the proteomes of T0 and -10^{o}C for 24 hours were not different, the few individual proteins that displayed significant differences in abundance between the two growth temperatures over 24 hours yield insight into the effects of temperature alone (as time is held constant). Effects of sub zero exposure time on *Cp34H* was also explored through the comparison of proteomes harvested at different timepoints in the -10^{o}C cell incubation. Unfortunately, *Cp*34H cells incubated at -1^{o}C for 8 weeks for proteomic analyses were compromised and could not be analyzed.

Temperature Effects: The NMDS analysis demonstrates that T0 and -10^{o}C 24 hours did not yield different proteomes (Figure 3), although a few individual proteins had statistically different abundances after 24 hours (61 total; 11 higher abundance, 37

decreased abundance after 24 hours at -10°C). In order to compare how the proteome changes in 24 hours at -1°C versus -10°C we compared the lists of proteins that were significantly different over 24 hours at the given temperature. This comparison revealed opposing quantitative trends of 8 proteins that result from a variable temperature (Table 2). Ton-B dependent receptors, RNA polymerase sigma factor, D-alanine—D-alanine ligase and a few conserved proteins increased in abundance when at -1°C for 24 hours and decreased in abundance when at -10°C (Table 2). On the other hand, no proteins presented the opposite trend: decreases in abundance after 24 hours at -1°C and increases in abundance after 24 hours at -10°C. A total of 5 proteins increased in abundance when held at -10°C for 24 hours, but did not change in concentration when held at -1°C, suggesting active synthesis over the 24-hour incubation at -10°C (Table 3). There were 33 proteins that are actively turned over, or degraded, after 24 hours at -10°C, yet were detected with same abundances when held at -1°C. Most of these 33 proteins can be categorized into 3 groups: cell envelope proteins, peptidase activity, and hypothetical proteins of unknown function (Table S2).

Time Effects: In order to understand proteomic adaptations of sustained exposure to subzero temperatures, *Cp*34H was incubated for 8 weeks at -10°C. As stated earlier, the NMDS analysis revealed that the overall proteome at T0 was similar to the proteome at -10°C for 24 hours (Figure 3). Therefore, to examine the effect of time alone on the proteome of *Cp*34H at -10°C, we looked at the proteins that were significantly different in abundance between 24 hours and 8 weeks at -10°C. Timepoint zero (T0) was not examined with respect to 24 hours, since those differences were previously discussed to understand the transition to -10°C ice. A total of 54 proteins significantly increased in abundance from 24 hours to 8 weeks at -10°C, suggesting that these proteins were newly synthesized and retained over the long period of time at -10°C. These proteins cluster into several Gene Ontology categories, including chemotaxis, GTP binding, nitrogen compound biosynthetic processes and nucleotide binding. These proteomic analyses revealed 43 proteins that were present at significantly lower abundance after 8 weeks of time at -10°C. Three Gene Ontology categories were significantly affected: the flagellum cellular component, metal ion binding molecular function, and the regulation of transcription biological process.

The samples from the -1°C incubation for 24 hours and the -10°C incubation for 8 weeks encompass the environmental transition from liquid water to ice over long periods of time and therefore represent the two biological states that are of greatest interest for this study. Incubation at -1°C for 24 hours could be viewed as a control state, representing the ideal biological functions of Cp34H after supplied adequate nutrition for 24 hours at a subzero temperature. The notable environmental and physiological changes are reflected in the NMDS analysis, where these two treatment groups show the greatest dissimilarity in proteomic profiles (Figure 3). Comparative analysis of protein abundance before (-1°C 24 hours) and after ice immersion (-10°C 8 weeks) identified 87 proteins with significantly enhanced abundance, and 137 proteins that showed significantly reduced abundance (fold change represented as $\log_2$ treatment/control; Dataset S1). Of the 224 proteins that were present at significantly

different abundances, 44 are described as having a hypothetical function and therefore cannot be further commented on in this study. Their presence at these temperatures does highlight them as a potential avenue for further investigation with molecular techniques.

Enrichment analysis (using DAVID v. 6.7) revealed that the biological processes enriched in the 137 lower abundance proteins at -10°C for 8 weeks compared to -1°C for 24 hours included homeostatic process, cell motion, cellular homeostasis, flagellum organization, ion homeostasis, transcription, and ciliary or flagellar motility (Table S2). Proteins found at higher abundance at -10°C after 8 weeks were enriched in the processes of response to DNA damage stimulus, DNA repair, ATP and helicase activity, chemotaxis, and locomotory behavior (Table S3).

Discussion

The metabolic activity of psychrophilic bacteria at subzero temperatures has been previously reported (e.g. Dieser et al. 2003; Junge et al. 2006; Mykytczuk et al. 2013), however, this is the first study that uses proteomics to elucidate some of the molecular mechanisms behind the ability of these bacteria to metabolize during extended periods of time at cold temperatures in saline ice. By combining metabolic assays (DNA and protein metabolism) with discovery-based proteomics, we provide direct evidence of metabolic pathways that allow *Colwellia psychrerythraea* to survive at subzero temperatures. Understanding the functionality of these systems will be instrumental in understanding their role in an ecosystem, especially in the context of global climate change. In order to mimic *in vitro* salt conditions found in the actual marine sea ice environment where *Colwellia* species are abundant (Junge et al., 2011), our incubations were conducted in ASW saline ice and did not contain added nutrients (with the exception of leucine). The biotic machinery and mechanisms that enable survival at subzero temperatures are challenging subjects to study, however, as we show here, tools such as tracking metabolism and proteomics can provide sensitive means of investigating and understanding low temperature growth and survival.

Colwellia psychrerythraea is capable of protein and DNA synthesis at subzero temperatures over an extended time period, as demonstrated by thymidine and leucine incorporation in ASW (liquid and ice). This is in agreement with previous reports of subzero growth and motility in marine broth suspensions (Huston et al., 2000; Junge et al., 2003; Marx et al., 2009). Our leucine incorporation results showed protein synthesis in *Cp*34H down to -10°C over the course of 8 weeks. These results are consistent with Junge et al (2006) where active leucine incorporation was demonstrated down to -20°C in *Cp*34H. Maximum [3H]-leu incorporation occurred at the 24 hour time point for all temperatures and was greatest in the -1°C treatment and lowest at -10°C. The decrease in *Cp*34H's metabolic rate with decreasing temperatures is consistent with general biological mechanisms and previous findings in psychrophilic bacteria (e.g., Junge et

al., 2006; Piette et al., 2011; Mykytczuk et al., 2013). At very low temperatures, low rates of metabolism may offset cellular and macromolecular damage in permafrost bacteria (Dieser et al., 2013) thereby allowing for survival over prolonged time frames (Amato and Christner, 2009). Cells exhibited continued high protein synthesis rates at all temperatures, indicating growth metabolism (according to definitions in Price and Sowers, 2004).

Thymidine incorporation assays on *Colwellia psychrerythraea* suggested DNA replication down to -10°C in ASW, signifying active genome replication and/or repair (Fuhrman and Azam, 1980). This is consistent with the results of Christner (2002) who found that the psychrophile *Psychrobacter sp*. maintained DNA synthesis for more than 23 weeks at -15°C. Similar to the trends seen in the [3H]-leu incorporation data, the lowest amount of thymidine incorporation occurred at -10°C in *Cp*34H and the highest at -1°C, implying a slowed metabolic rate as temperature decreased. Though our values for [3H]-thy incorporation at -10°C were lower than those previously reported in *Psychrobacter sp*. and *Arthrobacter sp.* (both from glacial ice) for -15°C (Christner, 2002), they follow the same trend as the [3H]-leu incorporation at all temperatures and thus demonstrate active DNA synthesis over the course of the 8 weeks. We suggest that cells in our saline ice samples responded differently to the cultures reported by Christner (2002), which were incubated in distilled water ice. This explanation is consistent with the microscopic observation of healthy cells during the entire course of the experiment, the protein synthesis rates discussed above, and the suite of proteomic data that indicate growing cells with moderate cell stress and no indication of a cessation of DNA synthesis pathways. Therefore, we suggest that further investigation and method development are required to determine the best protocol for measuring [3H]-thy incorporation in a saline ice environment.

Proteomics of *C. psychrerythraea* from four different treatment conditions yielded a total of 1763 identified proteins, encompassing a wide variety of physiological functions. Effects of temperature were evaluated by examining the expressed proteome of *Cp*34H after 24 hours at -1^{o}C and -10^{o}C. In addition, the effects of longer incubation times in ice on the expressed proteome were evaluated by comparing the proteomes after 24 hours and 8 weeks at -10^{o}C. Further, a direct comparison between bacteria held at -1°C for 24 hours and -10°C for 8 weeks revealed an exaggerated proteomic response of *Cp*34H under conditions that allow DNA synthesis and metabolic activity in -10^{o}C ice. This comparison of two proteomes after varying time and temperature regimes provided the statistical significance to adequately evaluate biological processes and metabolic functions *Cp*34H employs to maintain metabolic activity during sustained incubation in-10^{o}C ice.

Effects of temperature on Cp34H proteome over 24 hours

The primary responses observed in the proteome shifts between -1^{o}C and -10^{o}C are

affiliated with cell surface, substrate detection proteins. For example, methyl-accepting chemotaxis protein was newly synthesized after 24 hours at -10°C, whereas at -1°C, the abundance of this protein remained the same. Chemotactic proteins, such as these, appear to be first responders to extracellular environmental changes, suggesting that they are essential to bacteria for deciphering how to adapt cellular protein machinery. As few changes occurred in the proteome after 24 hours at -10°C, the increase in abundance of this chemosensing protein highlights its ability to respond even though enzyme kinetic rate is halved at -10°C relative to -1°C, based on the Arrhenius equation. In addition, decreases in growth temperature and the transition to ice decreases the need for *Cp*34H to maintain and utilize ton-B receptor proteins (Table 2). This likely results from the ATP requirement of these membrane proteins to transport substrates into the cell. Although leucine incorporation data demonstrates that *Cp*34H can bind small molecules to the cell at -10°C, the active transport by ton-B receptor proteins into the cell is decreased (see Iron metabolism section in Discussion). Substrates could still be transported by porins on the membrane surface, although at a slower rate.

Effects of time on Cp34H proteome at -10°C

Although *Cp*34H was incubated in ice at -10°C for 8 weeks, leucine incorporation proceeded and new protein synthesis occurred from 24 hours. The significant increase in abundance of proteins involved in chemotaxis suggests again that *Cp*34H must be constantly monitoring changes in the extracellular environment. Methyl-accepting chemotaxis protein was also determined to be newly synthesized after 24 hours of incubation at -10°C compared to -1°C. The new synthesis of these chemosensing proteins from T0→24 hours→8 weeks suggests that they result from the -10°C temperature and icy environment rather than the length of incubation time. After 8 weeks of time, intracellular signaling pathway proteins, such as those involved in GTP binding, were newly synthesized in the 8 week time course. Although the cells are in ice, the intracellular matrix is likely fluid, allowing diffusion, solute transport, and cell signaling. This intracellular communication likely directs the protein degradation pathway leading to decreased flagellum complexes over longer periods in time as *Cp*34H detects that the external environment is ice and therefore not a viable place for flagella-based movement. More experiments are necessary to determine all of the effects of time on *Cp*34H.

Mechanisms allowing survival in ice

The greatest proteomic adaptations are reflected in the NMDS analysis, where two treatment groups show the greatest dissimilarity in proteomic profiles. The contrast between cells grown at -1°C for 24 hours and cells grown at -10°C for 8 weeks emphasizes the physiological changes behind *Cp*34H's acclimation to and survival in ice-bound conditions relative to more standard conditions that allow normal metabolism and growth at subzero temperatures. These physiological changes encompass a variety of processes, providing insight into the diverse mechanisms necessary to maintain life in ice. We discuss some of these processes in detail below. ~~By comparing~~

~~the expressed proteome after *Cp*34H has been provided with and incorporated leucine and is replicating DNA for 24 hours to the proteome after 8 weeks of acclimation time to -10°C, we can comment on the enzymatic ability of a replicating and metabolizing cell line to a cell line that is maintaining survival over 8 weeks.~~

DNA repair

DNA repair proteins were found at higher abundance in *Cp*34H at -10°C after 8 weeks of incubation compared to -1°C after 24 hours, indicating potential DNA damage during bacterial exposure to ice. Some of these proteins are associated with the stress response to DNA damage, as illustrated by their contribution to the enrichment of the process response to cellular stress (including putative transcription accessory protein, DNA helicase II, exodeoxyribonuclease III, DNA mismatch repair protein MutS, DNA ligase NAD-dependent). Proteins involved in DNA repair were also enriched in the set of higher abundance proteins. The greater need for DNA repair may result from less new DNA being synthesized at -10°C, based on the [3H]-thy incorporation data. Four proteins identified at increased abundances after 8 weeks at -10°C are involved in DNA repair and maintenance (GTP-binding protein HflX, tRNA delta(2)-isopentenylpyrophosphate transferase MiaA, DNA mismatch repair protein MutS, PII uridylyltransferase GlnD). The increased abundance of MutS, a protein involved in mending point mutations in DNA, suggests that after 8 weeks at -10°C *Cp*34H maintains the integrity of its genome by combatting DNA damage post-replication (Acharya et al., 2003). DNA repair mechanisms have been observed to be at work in ancient permafrost bacteria (Johnson et al., 2007) and in bacteria subjected to ionizing radiation (Dieser et al., 2013), however this current study is the first to reveal which proteins and which repair mechanisms are predominantly used. The trade-off between DNA repair and synthesis warrants further investigation since many taxonomic groups of psychrophilic permafrost bacteria (including Proteobacteria, the phylum that contains *Colwellia*) are able to synthesize DNA after extended exposure to subzero temperatures, some down to -20°C (Tuorto et al., 2014). The decreased abundance of repressor LexA, a protein involved in muting SOS regulons and depressing the repair signal strengthens our proposal of a concerted effort by *Cp*34H to tightly regulate DNA repair. RecA, a protein strongly affiliated with the function of LexA in the DNA repair system, was present with the same measured relative abundance across all temperatures and time points. Compared to LexA, RecA may have a different turnover rate or be differentially regulated (i.e. post-translationally modified).

The inclusion of two DNA-binding cold shock proteins amongst our 15 most abundant proteins identified in *Cp*34H provides insight into ways that this true psychrophile may withstand low temperature conditions (Table 1). Cold-shock proteins are a class of proteins that assist in maintaining cellular function during shifts to lower temperatures (Phadtare et al., 1999). These proteins are highly conserved and have

been found in many prokaryotic and eukaryotic organisms and are well studied in mesophilic species such as *Escherichia coli* (Phadtare et al., 1999; Ermolenko and Makhatadze, 2002). Evidence for cold shock proteins at the genomic level has been reported for *C. psychrerythraea* (Methe et al., 2005) and in other species of psychrotolerant bacteria (Ayala-del-Rio et al. 2010; Mykytczuk et al., 2013). The presence of these cold-shock DNA binding proteins under all experimental conditions (Dataset S1) indicates that they represent an essential function and that they routinely facilitate protein synthesis at low temperatures in this species..

Transcription and translation

Many of the proteins involved in response to incubation at -10°C for 8 weeks are associated with protein synthesis. Some of these proteins are helicases, which are often implicated in the unwinding of DNA for transcription, translation, and replication. Since DNA replication levels were lower at -10°C, the increased abundance of helicases were likely involved in increasing transcription, translation, and repair. Some of the helicases found at greater abundance in the -10°C treatment were DNA helicase II, SNF2 family protein, RNA helicase DeaD, and ATP-dependent helicase DEAD-box family. Additionally, proteins involved directly in translation were found at higher levels in the lower temperature treatment, supporting the hypothesis of translation and protein synthesis to support mechanisms of cold tolerance. These proteins include ribosomal protein S12, tRNA delta(2)-isopentenylpyrophosphate transferase, and tRNA modification GTPase TrmE. A putative heat shock protein 70 family protein was also found at significantly greater abundance in this treatment group, illustrating a potential increase in enzymes responsible for protein folding, maintenance, and stabilization.

Other proteins that were constitutively expressed, but not found at differential abundances between treatments, include those that are known to specialize in protein folding in cold-adapted organisms (Feller, 2003). These proteins included multiple isoforms of both FKBP-type peptidyl-prolyl cis-trans isomerases and peptidyl-prolyl cis-trans isomerases as well as a trigger factor. Some of the greatest challenges for life at subzero temperatures include protein synthesis and stability (reviewed in Feller, 2013), yet the proteins in psychrophiles continue to function and allow for growth even at these low temperatures. The ability to maintain protein function at low temperatures is derived from an increased abundance of specific proteins that maintain function as temperature decreases as well as changes to protein structure that allow for increased flexibility/decreased stability (Feller, 2013). Evidence for increased protein flexibility at cold temperatures has been documented at the genomic level in psychrophiles (Ayala-del-Rio et al., 2010; Mykytczuk et al., 2013) and we have now found evidence of changes in abundance of specific cold adapted proteins as well as proteins that increase stability during transcription and translation in response to 8 weeks of incubation at -10°C in *Cp*34H.

Flagellar proteins and motility

The lower abundance of flagellar proteins at -10°C 8 weeks suggests that *C. psychreythraea*'s motility was affected by cold incubation. Flagellar proteins were well-represented in the set of lower abundance proteins in response to cold incubation (Table S2). *C. psychrerythraea* is a flagellated organism, previously identified to exhibit motility in glycerol-amended marine broth down to -10^{o}C, but motility ceases with ice immersion (Junge et al., 2003). The average speed recorded at -10°C for *Cp*34H was 28 μm s^{-1}, nearly half the speed observed at -5°C (though still comparable to speeds of cultured enteric and marine bacteria at mesophilic temperatures (Junge et al. 2003)). In agreement with these earlier microscopic findings, our proteomic examination revealed ten flagellar proteins that were at lower abundance in *Cp*34H cells held at -10°C for 8 weeks compared to -1°C for 24 hours (Figure 4A). The basal body flagellar biosynthetic protein (FlhF; Figure 4A) was 2-fold lower at -10°C and has been noted to be a critical protein for regulating the ultimate transcription of other flagellar proteins (Niehus et al., 2004). Previous studies of flagellar assembly have temporally categorized the genes into early, middle and late transcriptional classes in *E. coli* and *Salmonella* (Chilcott and Hughes, 2000). Of the ten lower abundance proteins in *Cp*34H at -10^{o}C, 3 are considered to be translated from "late genes" by this classification method (FlaG and flgK and FliS) and six are considered translated from "middle genes" (FliM, FlgE, FliK, FlgK,and FlgH) (Figure 4B). The decreased abundance of these flagellar proteins may be a reflection of overall decreased metabolism in *Cp*34H at -10°C.

It should be emphasized that although several flagellar assembly proteins were affected by -10°C after 8 weeks, they were still identified in cells maintained at -10°C. This is consistent with previous findings that *Cp*34H retains limited motility when immersed in liquid (Junge et al., 2003). Four enzymes related to chemotaxis-locomotion were present in increased abundance in the -10°C 8 weeks culture (Table S3). Several of these are recognized as methyl-accepting chemotaxis proteins (M*CP*) that are known to help bacteria decipher the medium in which they reside. As transmembrane sensors, M*CP*s may provide a constant feedback loop to *Cp*34H so it can determine if the remaining locomotion machinery (e.g., flagellum FlaG) should be assembled. In addition, one flagellum-related protein was more abundant at -10°C 8 weeks: FliL (Figure 4A and B). Although little is known about FliL, it is proposed to participate in the coupling of MotB with the flagellar rotor in an indirect fashion and is part of the basal flagellum complex (Suaste-Olmos et al., 2010). Similarly, a regulator of flagellar synthesis (FliA) had increased expression at the transcriptional level in response to -15°C in the permafrost bacterium *Planococcus halocryophilus* (Mykytczuk et al., 2013). These proteomic findings indicate that *Cp*34H does not cease motility in -10°C ice over 8 weeks, instead, to reduce the energy requirements for the maintenance of their motility apparatus, our findings suggest that the cells use chemo-sensing proteins (M*CP*) to monitor their surroundings and do not assemble the flagellum complex when

exposed to ice.

Membrane flexibility

Multiple enzymes identified as polyunsaturated fatty acid (PUFA) synthases (pfaC, pfaA, and pfaD) were identified in the dataset, but only pfaC was found in greater abundance after the 8 weeks of incubation at -10°C (0.58-fold, z-statistic = 4.22). We expected a greater change in PUFA-associated proteins because amendments in PUFAs have been associated with the maintenance of cellular flexibility and solute transport at subzero temperatures (Nichols et al., 1997; Huston et al., 2004). The *Cp*34H genome contains multiple genes that code for enzymes that would increase membrane fluidity (Methe et al., 2005). In *Planococcus halocryophilus*, a bacteria found in permafrost, Mykytczuk et al. (2013) observed an increased expression of the mRNA for two enzymes involved in membrane lipid metabolism in response to -15°C: cardiolipin synthetase and a 3-oxoacyl-(acyl-carrier-protein) reductase. The lack of response that we observed in the membrane flexibility pathway may be because we were comparing bacterial proteomes at two subzero temperatures (i.e. membrane alterations are required at both -1°C and -10°C), whereas Mykytczuk et al. (2013) compared transcriptomes between bacteria grown at 25°C and -15°C.

Although pfaC was at greater abundance at -10°C after 8 weeks, it was still only 1/5th as abundant on average as pfaA (39 spectral counts compared to 240). The transcriptional start site for genes pfaA-C is pfaA (Allen and Bartlett, 2002). We suggest that constitutively high levels of pfaA are part of the initial response during a need to alter membrane saturation or the turnover of this gene product may be slow in this psychrophilic bacterium. To date, differences between polyketide PUFAs are unknown, however these proteomic findings suggest that pfaA plays a larger role in regulating membrane flexibility at extreme low temperatures. Interestingly, the 3-oxoacyl-(acyl-carrier-protein) and 3-ketoacyl-(acyl-carrier-protein) reductase proteins are involved in the first steps of fatty acid biosynthesis and were identified in all samples, but neither showed differential abundance between treatments.

Iron metabolism

After incubating *Cp*34H at -10°C for 8 weeks, the proteins that facilitate the uptake of the more soluble form of iron, ferric iron, were at lower abundance compared to *Cp*34H at -1°C for 24 hours (e.g. bacterioferritin and TonB dependent proteins; Dataset S1). Six TonB dependent proteins were less abundant (between 1.2 - 2.5 fold). TonB-dependent proteins are perched on the outside of bacterial membranes, responsible for energy-dependent uptake of substrates through channels, such as porins, from the surrounding environment into the cells. Specifically, TonB-dependent transport is recognized to bring siderophores (Fe-binding organic ligands) and heme complexes into the cell in many bacteria species (Clarke et al., 2001). As a result of the lower protein abundance of both siderophores and TonB-dependent receptors, we propose *Cp*34H does not utilize this

energy dependent Fe-transport system since the production and release of siderophores into brine channels is likely a costly and unprofitable process.

On the other hand, ferrous iron transporters and multiple heme biosynthesis proteins were at greater abundances when the cells were maintained at -10°C for 8 weeks compared to -1°C for 24 hours. In general, ferrous iron only dominates anaerobic systems. It is possible that, by 8 weeks of time, the system was depleted in oxygen and *Cp*34H had acclimated to use the ferrous transport system, followed by the intracellular creation of stable heme proteins for electron transfer and ATP generation (Mobius et al., 2010). Rysgaard and Glud (2004) demonstrated that as seawater turns to ice, oxygen bubbles can get trapped in the ice making them unavailable to support aerobic respiration. Although we do not have data on the dissolved oxygen content within the liquid brine inclusions that harbored the cells in the ice in these experiments, this hypothesis would support the observed lower abundances of glutathione reductase and thioredoxin at -10°C as these oxidative-stress related proteins are not necessary in an anaerobic system, as well as the hypothesis of increased denitrification activity (see below). Piette et al (2011) also noted a decreased abundance of oxidative-stress related proteins and downregulation of Fe-uptake enzymes in the marine bacterium *P. haloplanktos* at low temperatures (4^{o}C), suggesting the changes were a result of the suppression of oxidative metabolism as well as the decreased abundance of a variety of enzymes that require iron.

Nitrogen metabolism

There is evidence for trade-offs in nitrogen metabolism in the cells incubated at -10°C for 8 weeks, with a shift towards denitrification over nitrogen uptake. Both the periplasmic nitrate reduction enzyme (Nap), essential for nitrate uptake, and the nitrogen regulatory protein PII were at lower abundance at -10°C after 8 weeks. Low temperatures (below the temperature for optimum growth rate) are known to decrease nitrate uptake across bacteria species, including psychrotolerant species from Antarctic lake sediment, perhaps since nitrate uptake is an ATP-dependent process (Reay et al., 1999). Nitrogen is critical to bacteria in order to continue to replicate and synthesize proteins. From our [3H]-leu and [3H]-thy experiments it is evident that *Cp*34H is capable of DNA replication and repair and protein metabolism down to -10^{o}C. Even though we observed decreased abundances of nitrogen uptake proteins, protein synthesis could still be occurring through use of nitrogen reserves and uptake of nitrogen-based leucine and thymidine. There is also genomic evidence in *Cp*34H for genes that code for proteins that degrade polyamides to increase nitrogen reserves, which may be a unique adaptation to a cold environment (Methe et al., 2005).

In contrast, the denitrification regulatory enzyme (NirQ) was at greater abundance at -10^{o}C. Evidence of bacterial denitrification in sea ice has been observed in ice cores from multiple locales (Kaartokallio, 2001; Rysgaard et al., 2001; Rysgaard et al., 2008). Brine pockets within sea ice provide an ideal habitat for denitrification to

occur during anoxic conditions and high nitrate and organic carbon concentrations (Rysgaard and Glud, 2004).. In general, denitrification occurs where oxygen is depleted, a condition also suggested by our iron metabolism data, and is estimated to account for 27% of the bacterial activity in ice-covered arctic areas (Rysgaard et al., 2008). These findings suggest that denitrification becomes an increasingly important metabolic process in *Cp*34H after long periods at subzero temperatures since nitrogen and oxygen may be limited due to environmental restrictions.

As many questions as we were able to answer with this detailed analysis, there are still many more that warrant further investigation. There are many environmental factors that were different between our two compared treatments of -1°C 24 hours and -10°C 8 weeks. Future studies should focus on the specific mechanisms utilized by psychrophiles to adapt to the physical changes of exposure to ice, low temperature, increased salinity, decreased oxygen, and changes in nutrient availability as their medium transitions from liquid to ice.

Conclusions

The proteomic analysis of *C. psychrerythraea* revealed a number of physiological mechanisms that are employed to maintain bacterial function at subzero temperatures in ice. These included a reallocation of resources from DNA binding to DNA repair, a decreased focus on motility, and changes to iron metabolism, nitrogen metabolism, and cellular membrane structures. Some of the proteomic responses observed in this study were already suspected to be important in cold-active bacteria given the expansion of these gene families within the *Cp*34H genome (Methe et al., 2005), however, in this study we provide evidence of active protein translations in response to a colder environment. Even though growth and metabolism in psychrophilic bacteria are significantly slowed at cold temperatures (Junge et al., 2006; Piette et al., 2011; Mykytczuk et al., 2013), many species are able to survive for extended periods in ice (e.g., Amato et al., 2010; Dieser et al., 2013). The mechanisms we have uncovered suggest, in part, how the maintenance of this metabolic activity is possible.

Experimental Procedures

Bacterial strain, culture conditions and cellular preparations

Colwellia psychrerythraea strain 34H (ATCC No. BAA-681; GenBank Accession No. AF396670) used in this study was originally isolated from Arctic marine sediments (Huston et al., 2000) and represents a genus common in sea ice (Bowman et al., 1998). Cells were cultured from frozen glycerol stocks by incubating at -1°C with daily inversion in half-strength Marine 2216 broth (DIFCO laboratories, Detroit, MI) until early stationary growth phase (2 weeks or less). Prior to the initiation of the experiments, cells were harvested by centrifugation (2800x*g;* 20 min; 4 °C). After washing with cold (-1°C), 0.2 µm filtered, artificial seawater (ASW: 0.1M NaCl, 0.01M KCl, 0.06M MgCl2, 8mM

$MgSO_{47}H_20$, 5mM TAPSO [3-[N- tris(hydroxymethyl) methylamino]-2-hydroxypro-pane-sulfonic acid], pH 7.5), cells were resuspended in ASW (-1°C) with 1.5 g C ml^{-1} extracellular polysacharide substances (EPS; obtained from *Cp*34H cells using methods described by Huston et al., 2004) to an OD600 nm of 0.2 corresponding to an average ($\pm$standard deviation) cell count of 5.7E7 $\pm$ 1.1E7 cells ml^{-1}. Triplicate 100 µl aliquots of cell suspensions were fixed in 2% formaldehyde, and stored at 4°C for abundance analysis by epifluorescence microscopy as described by Junge et al (2004b). For [3H]-leucine and [3H]-thymidine incubation experiments, 500 µl aliquots of the cell suspension were placed into 1.5-ml Eppendorf tubes for immediate use. An 8-week incubation time was chosen to represent and determine proteome changes over prolonged exposure in ice (versus short-term exposure over 24 hours) and thus represent how cells that remain in sea ice after the initial freeze in fall acclimate over time to that environment in the winter.

[3H]-leucine and [3H]-thymidine incorporation assays

Bacterial metabolic activity was measured using leucine ([3H]-leu) and thymidine ([3H]-thy) incorporation assays, following methodologies based on the analysis of bacterial activities in freshwater ice (Christner, 2002) and saline ice (Junge et al., 2006). To determine rates of [3H]-leu incorporation and [3H]-thy incorporation in the prepared cell suspensions, time-course experiments were conducted at temperatures of -1°C, -5°C, and -10°C and sampling occurred at 0, 1, 12, and 24 hour, and 1, 2, 4 and 8 week time points. Triplicate negative (killed) controls were completed at each temperature by adding 100 µl of 50 % trichloroacetic acid (TCA; 4°C) to control tubes prior to tracer addition. Methods described by Junge *et al*. (2006) were primarily followed, with the exception of modifications including: 1) samples were placed at their intended temperatures immediately after the addition of the radiolabel tracer, not flash frozen with LN_2 (see Text S1), 2) three ethanol washes were performed during the processing stage, and 3) in addition to the [3H]-leu incorporation assay to measure metabolic activity based on protein synthesis, [3H]-thy incorporation was also measured to investigate rates of DNA synthesis.

Rate calculations

Incorporation rates of radioactivity were calculated from the linear portions of the time-course curves for [3H]-leu (up to the 24 hour time point when saturation was reached) and scaled to bacterial numbers. In order to cross-compare with literature values of bacterial metabolic rates, rates were converted from nmol [3H]-leu incorporated per unit time to grams of leucine carbon incorporated per grams of bacterial carbon per hour, using common conversion factors (7.3 mol% = fraction of leucine in protein, 131.2 g mol^{-1} = molecular weight of leucine, 0.86 = ratio of cellular carbon to protein; Kirchman, 1993) and assuming 65 fg C $bacterium^{-1}$ (as in Price and Sowers, 2004; Junge et al., 2006).

Proteomic sample preparation

For proteomic interrogations, biotriplicate samples of *Cp*34H cells were collected at four time points: T0 (the starting cell culture prior to leucine additions), after 24 hours at -1°C, as well as from cells incubated at -10°C for 24 hours and 8 weeks. These time points were chosen for their potential to capture the proteomic shifts that occur when cells that inhabit a liquid state are frozen into ice, similar to what happens during the fall to winter transition in the Arctic. Cellular preparations for proteomic analyses were completed as follows: cell pellets were lysed in 100μl 50mM ammonium bicarbonate with 6M urea using a titanium micro-probe sonicator (Branson 250 Sonifier; 20kHz, 10 x 10s on ice). Protein disulfide bonds were reduced with 2.5μl 200 mM tris(2-carboxyethyl)phosphine (37°C, 1 hr) and alkylated with 20μl 200 mM iodoacetamide (20°C, 1 hr, dark). Dithiolthreitol (20μl 200mM, 1 hr) was added to soak up remaining iodoacetamide. Samples were diluted with 800μl 25mM ammonium bicarbonate and 200μl methanol followed by digestion with Promega Trypsin (1:25; enzyme:protein) overnight at 37°C. Prior to mass spectrometry, samples were desalted using microspin C18 columns following manufacturer's guidelines (Nest group). Peptide concentrations were measured on each sample using the Thermo Scientific NanoDrop 2000/2000c Spectrophotometer. The peptide bond absorbance was monitored at 205 nm UV wavelength and samples were diluted to yield a final concentration of 100 µg protein ml^{-1}. Peptide samples were then frozen at -80°C until liquid chromatography tandem mass spectrometry analyses were performed (LC-MS/MS).

Mass Spectrometry

Based on peptide concentrations, a total of 1 µg of peptide digest in 10 µl of 5 % ACN, 0.1 % formic acid was sampled per LC-MS/MS analysis. Samples were separated and introduced into the mass spectrometer (MS) by reverse-phase chromatography using a 25 cm long, 75 µm i.d., fused silica capillary column packed with C18 particles (Magic C18AQ, 100 Å, 5; Michrom, Bioresources, Inc., CA) fitted with a 2 cm long, 100 µm i.d. precolumn (Magic C18AQ, 200Å, 5; Michrom). Peptides were eluted using an acidified (formic acid, 0.1 % v/v) water-acetonitrile gradient (5-35 % acetonitrile in 90 min). Mass spectrometry was performed on a Thermo Fisher (San Jose, CA) QExactive (QE). The top 20 most intense ions were selected for MS2 acquisition from precursor ion scans of 400-1600 m/z. Quality control (QC) peptide mixtures were analyzed every sixth injection to monitor chromatography and MS sensitivity. Skyline was used to determine that QC standards did not deviate >10 % through all analyses (Maclean et al., 2010). For quantitative analyses, biotriplicate samples from T0, -1°C 24 hours, -10°C 24 hours, -10°C 8 weeks were analyzed on the QE in technical duplicates using data-dependent acquisition (DDA), culminating in a total of 6 analyses per treatment. These 6 independent analyses per treatment provided a dataset from which statistical

confidence could be applied to determine which proteins were present in significantly increased or decreased abundance with respect to the alternate cell state using *QSpec* (see below).

Protein Database Searching and Mass Spectrometry Data Interpretation

All tandem mass spectrometry results were searched and interpreted with COMET (Eng et al., 2013). The protein database used for correlating spectra with protein identifications was generated by combining the latest release of *Colwellia psychrerythraea* proteins on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/; April 2013) consisting of 4,910 proteins, 50 common contaminants, and the QC peptides. COMET parameters included: reverse concatenated sequence database search, Trypsin enzyme specificity, cysteine modification of 57 Da (resulting from the iodoacetamide), and modifications on methionine of 15.999 Da (oxidation). Concatenated target-decoy databases searches were completed and minimum protein and peptide thresholds were set at $p > 0.95$ on ProteinProphet and PeptideProphet (Nesvizhskii et al. 2003). Protein identifications from the whole-cell lysates were accepted by ProteinProphet if the abovementioned thresholds were passed, two or more peptides were identified (PeptideProphet), and at least one terminus was tryptic (Keller et al., 2002).

This suite of proteomics data was further interrogated to determine relative quantities of proteins observed between culture conditions and time points. The common method of spectral counting was employed to determine relative protein abundance (Choi et al., 2008; Collier et al., 2010; Hoehenwarter and Wienkoop, 2010; Li et al., 2010). Spectral counting sums up the number of identified peptide tandem mass spectra resulting from a specific protein in order to estimate abundance of that protein relative to other proteins in the sample. Using QSpec to determine if proteins were significantly differentially abundant, six different significance analyses were completed to compare *Cp*34H between 2 culturing conditions and/or time points (e.g., T0/-10°C 24 hours and -1°C 24 hours/-10°C 8 weeks; Dataset S1) (Choi et al., 2008). QSpec was designed specifically for interpreting differences in protein populations determined from tandem mass spectrometry spectral counts. QSpec normalizes spectral count data based on protein length as well as by average spectral count across all proteins (Choi et al. 2008). QSpec is reported using a fold change difference in abundance on a log base 2 scale. This provides an easy way to examine the data because a reported positive fold change indicates significant increases in abundance and a negative fold change indicates significant decreases in abundance. A reported fold change of zero indicates no significant difference was measured between the treatments. Proteins were considered significantly reduced or enhanced in abundance if the reported z score was ≥ 2 or ≤ -2 and the fold difference observed was ≥ 0.5 or ≤ -0.5 (Choi et al., 2008).

A non-metric multi-dimensional scaling analysis (NMDS) was done on the log(x+1)-

transformed spectral count data for all proteins that had at least 2 spectral counts across all biological replicates (Dataset S1). One of the biological replicates for T0 was determined to be an outlier and was therefore excluded from this analysis. For the NMDS, a Bray-Curtis dissimilarity matrix was employed. ANOSIM was performed on the dataset normalized by row to find significant groupings based on temperature and incubation time. Both NMDS and ANOSIM were performed in R version 3.1.0 using the vegan package (Oksanen et al., 2013; R Core Development Team, 2013).

The Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7) was used to identify biological significance within the large dataset (Huang et al., 2009a; Huang et al., 2009b). Proteins that were determined to be at significantly greater or lower abundance in response to time and temperature (-1°C 24 hours versus -10°C 8 weeks) were examined by this functional annotation tool. All proteins identified across biological and technical replicates were used as the background gene list.

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TABLES

Table 1. Fifteen most abundant proteins identified among all experiments based on total spectral counts across biological and technical replicates with respective Gene Ontology categories. Percent total observed spectra is reported based on the total number of spectral counts for all proteins identified (915396).

Protein Name	Biological Process Main Role	Biological Process Sub Role	Total Spectral Counts	Percent Total Observed Spectra
Translation elongation factor Tu	Protein synthesis	Translation factors	26514	2.9
ribosomal protein S1	Ribosomal proteins: synthesis and modification	Protein synthesis	17861	2.0
ribosomal protein L7/L12	Ribosomal proteins: synthesis and modification	Protein synthesis	10827	1.2
chaperone protein DnaK	Protein folding and stabilization	Protein fate	10423	1.1
cold-shock DNA-binding domain protein	Adaptations to atypical conditions	Cellular processes	10270	1.1
alanine dehydrogenase	Amino acids and amines	Energy metabolism	9943	1.1
DNA-directed RNA polymerase, beta' subunit	DNA-dependent RNA polymerase	Transcription	9918	1.1
DNA-directed RNA polymerase, beta subunit	DNA-dependent RNA polymerase	Transcription	9877	1.1
translation elongation factor G	Protein synthesis	Translation factors	9517	1.0
cold-shock DNA-binding domain protein	Adaptations to atypical conditions	Cellular processes	9088	0.99
TonB-dependent receptor	Cations and iron carrying compounds	Transport and binding proteins	9001	0.98
ATP synthase F1, beta subunit	Energy metabolism	ATP-proton motive force interconversion	8515	0.93
polyribonucleotide nucleotidyltransferase	Degradation of RNA	Transcription	8161	0.89
ATP synthase F1, alpha subunit	Energy metabolism	ATP-proton motive force interconversion	8110	0.89
ribosomal protein L9	Protein synthesis	Ribosomal proteins: synthesis and modification	7459	0.81

Table 2. Proteins identified to significantly increase in abundance after 24 hours at -1°C and decrease in abundance after 24 hours at -10°C. No proteins were revealed to decrease in abundance after 24 hours at -1°C and increase in abundance after 24 hours at -10°C.

ID	Protein Description	T0 →24 hrs -1°C	T0 → 24 hrs -10°C
71282170	TonB-dependent receptor	up	down
71281574	TonB-dependent receptor	up	down
71282497	TonB-dependent receptor	up	down
71280628	TonB-dependent receptor	up	down
71278347	RNA polymerase sigma factor RpoS	up	down
71281200	D-alanine--D-alanine ligase	up	down
71279600	conserved hypothetical protein	up	down
71281256	conserved hypothetical protein	up	down
	NONE FOUND	DOWN	UP

Table 3. Proteins that significantly increased in abundance after 24 hours -10°C, but did not change in concentration when held at -1°C for 24 hours. * indicates proteins present at significantly different final abundances after 24 hours at -1°C or -10°C.

ID	Protein Description	T0 →24 hrs -1°C	T0 → 24 hrs -10°C
71279686	methyl-accepting chemotaxis protein/sensory box protein	--	up
71278541	molybdenum cofactor biosynthesis protein C	--	up
71278143	polyhydroxyalkanoate depolymerase, intracellular	--	up
71281317	Xaa-Pro aminopeptidase*	--	up
71277784	hypothetical protein*	--	up

LIST OF FIGURES

Figure 1. [3H]-leucine and [3H]-thymidine incorporation as a function of time to determine protein and DNA synthesis, respectively. Average values and standard deviations (represented by the error bars) are reported from biotriplicate experiments of *Cp*34H grown at -1°C (triangles), -5°C (crosses), -10°C (squares), and killed controls (circles).

Figure 2. Schematic of experimental conditions for proteomic analyses. Samples were harvested in biotriplicate for all timepoints (circles) at either -1^{o}C or -10^{o}C and bioduplicates for T0.

Figure 3. A non-metric multi-dimensional scaling analysis (NMDS) plot of all protein spectral count data (from proteins with >2 spectral counts) resulting from biotriplicate samples of 4 experimental conditions: T0 (closed triangles), -1°C 24 hours (closed squares), -10°C 24 hours (open square), -10°C 8 weeks (open diamond).

Figure 4. A) Proteins involved in flagellum assembly determined to be either significantly greater or lower abundance when *Cp*34H is maintained at -10°C for 8 weeks compared to *Cp*34H maintained at -1°C for 24hrs. B) Illustration of location of proteins involved in the assembly of flagella. Red indicates proteins observed to be at significantly greater abundance in -10°C after 8 weeks, Blue indicates proteins observed to be at significantly lower abundance at -10°C after 8 weeks, black complexes means the protein was observed but had no significant increase or decrease between the cells states, and black with no bold text is indicative of proteins that were not observed in this proteomic profile.

SUPPLEMENTAL DATA

Dataset S1. All proteins identified from 4 experimental conditions in biotriplicate analyzed using MS-based proteomics with corresponding spectral counts and QSpec output signifying if significant higher or lower abundance was observed between cell states examined.

Table S1. Average [3H]-leucine incorporation and [3H]-thymdine incorporation measurements from biotriplicate samples with respective standard deviations for all temperature conditions across 8 time points.

Table S2. Proteins that significantly decreased in abundance after 24 hours at -10^{o}C, suggesting turnover, but did not change in concentration when held at -1^{o}C for 24 hours.

Table S3. Gene Ontology categories of enriched biological processes in the 137 proteins showing decreased abundance after exposure to -10°C for 8 weeks . When multiple enriched biological processes shared all contributing proteins they were combined into one row and each process's p-value is reported if it differed from the others. Count: total proteins present with significantly lower spectral counts at -10°C 8 weeks (compared to all proteins) that correlated with the Biological Process; P-value: probability that the number of proteins identified in the biological process is significant with respect to the total number of proteins from the *C. psychrerythraea* proteome associated with that process.

Table S4. Gene Ontology categories of enriched biological processes in the 87 proteins showing increased abundance after exposure to -10°C for 8 weeks. When multiple enriched biological processes shared all contributing proteins they were combined into one row and each process's p-value is reported if if differed from the others. Count: total proteins present with significantly higher spectral counts at -10°C 8 weeks (compared to all proteins) that correlated with the Biological Process; P-value: probability that the number of proteins identified to increase in abundance in the biological process is significant with respect to the total number of proteins from the *C. psychrerythraea* proteome associated with that process.

Text S1. Details of experiments performed to test *Cp*34H response to flash-freezing prior to incubation at desired subzero temperature versus directly incubating at the desired temperature without flash freezing.

Figure S1. Average **[**3H]-leucine incorporation for biotriplicate samples with standard deviations of samples incubated for 24 hours after either being flash frozen (black bars) in liquid nitrogen prior to incubation or being placed immediately at the incubation temperature (gray bars; see Text S1).

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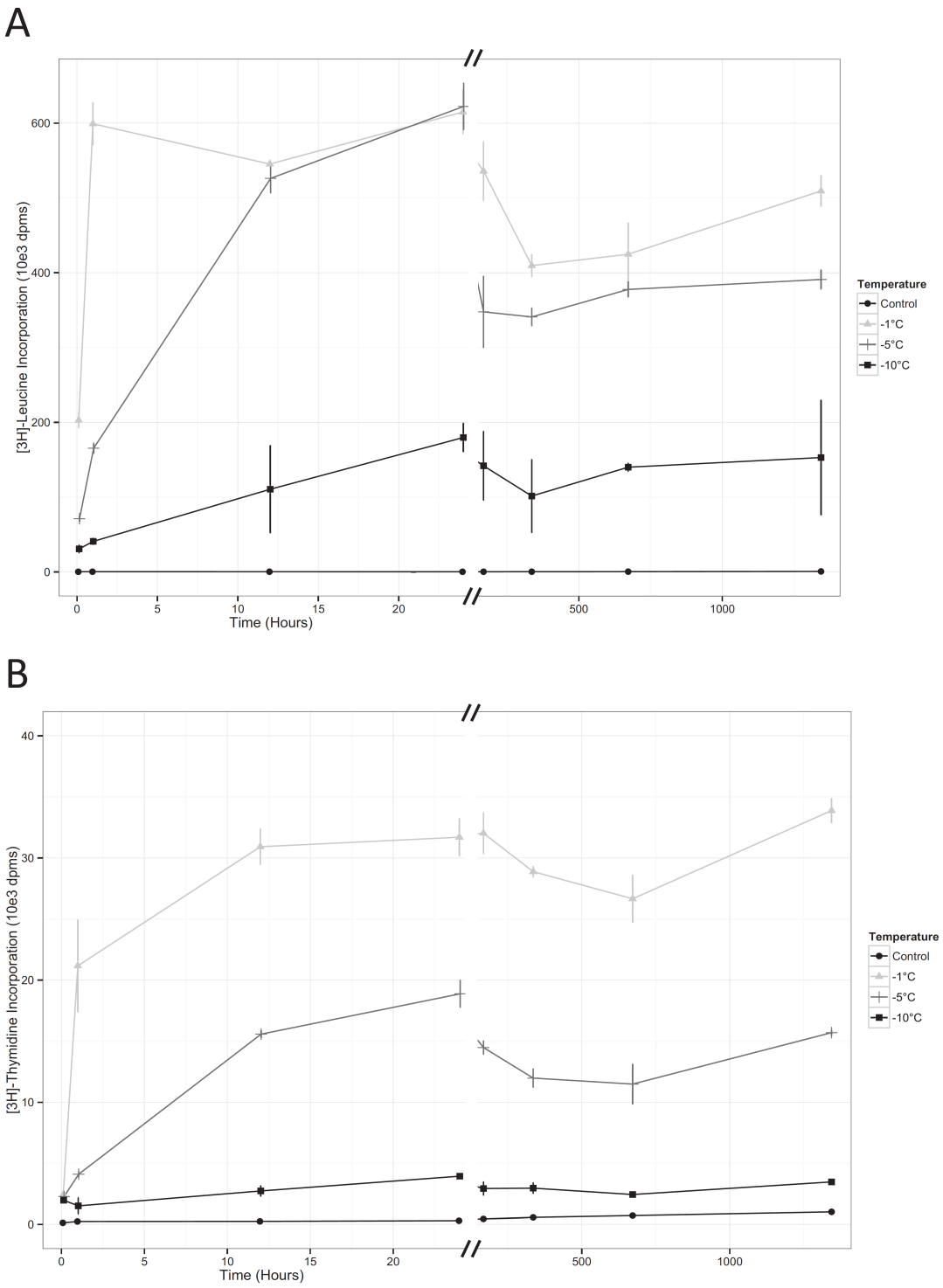

Fig. 1. A. [3H]-leucine and B. [3H]-thymidine incorporation as a function of time to determine protein and DNA synthesis respectively. Average values and standard deviations (represented by the error bars) are reported from biotriplicate experiments of *Cp*34H grown at −1°C (triangles), −5°C (crosses), −10°C (squares), and killed controls (circles).

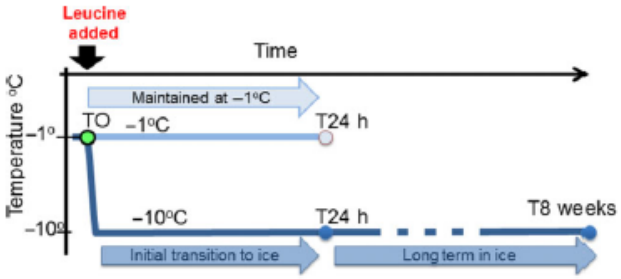

Fig. 2. Schematic of experimental conditions for proteomic analyses. All cells were initially grown in half-strength marine broth until early stationary growth phase (2 weeks or less), harvested by centrifugation and then transferred into artificial seawater (ASW) for experimental incubations at respective temperatures (–1°C or –10°C). Samples were harvested in biotriplicate for all time points (circles) at either –1°C or –10°C and bioduplicates for T0 (green circle).

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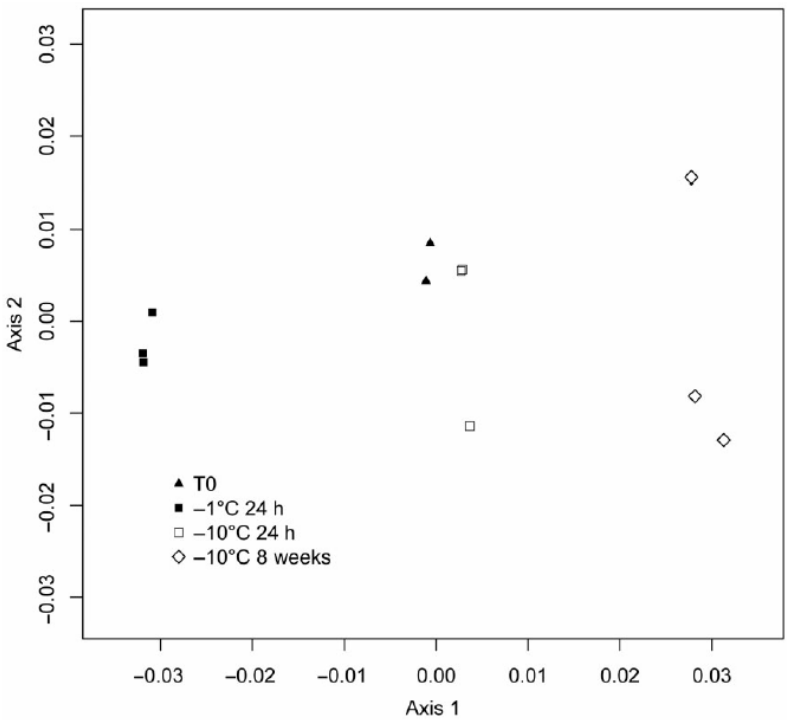

Fig. 3. A non-metric multi-dimensional scaling analysis (NMDS) plot of all protein spectral count data (from proteins with > 2 spectral counts) resulting from biotriplicate samples of four experimental conditions: T0 (closed triangles), –1°C 24 h (closed squares), –10°C 24 h (open square), –10°C 8 weeks (open diamond).

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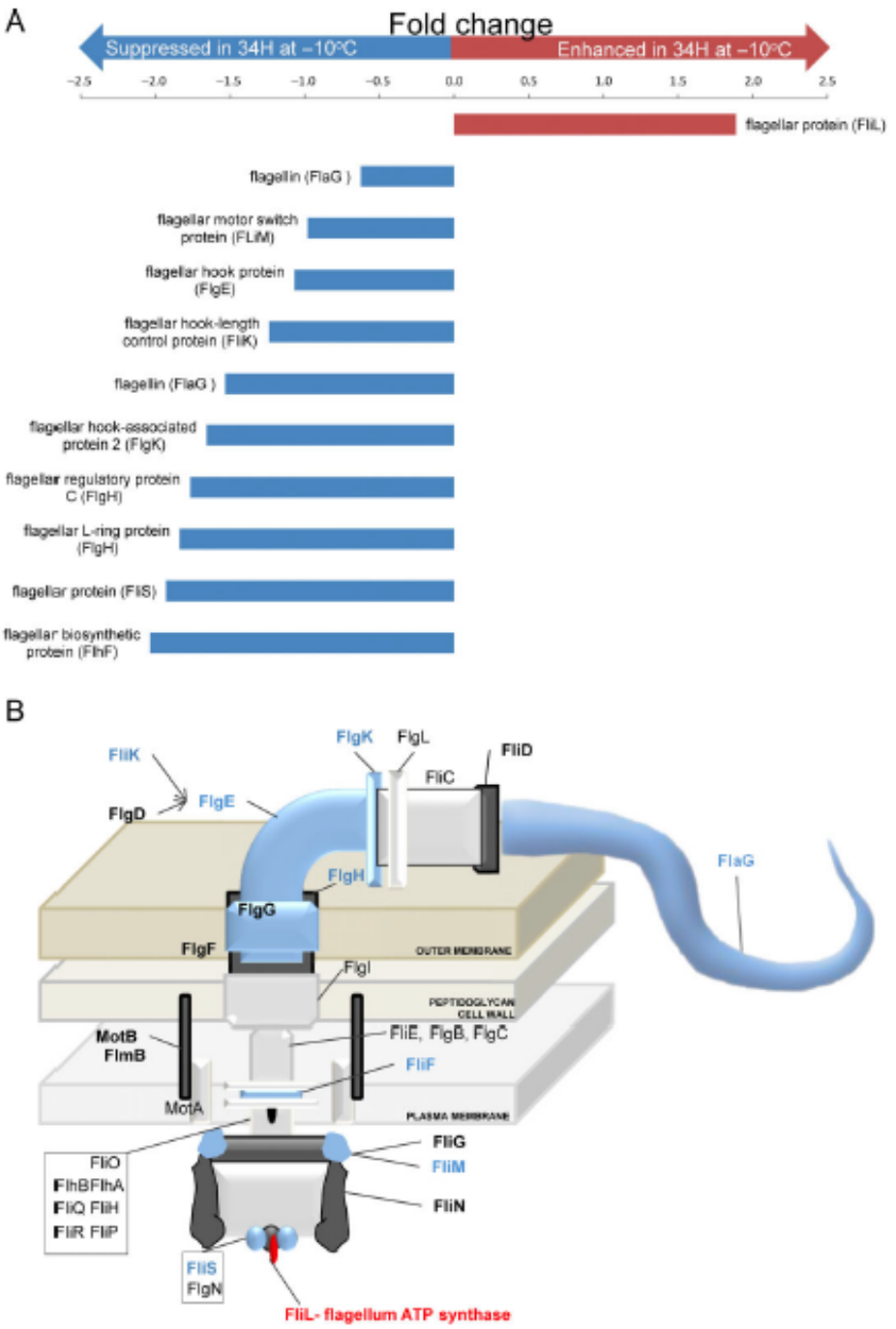

Fig. 4. A. Proteins involved in flagellum assembly determined to be either significantly greater or lower abundance when *Cp*34H is maintained at −10°C for 8 weeks compared with *Cp*34H maintained at −1°C for 24 h.
B. Illustration of location of proteins involved in the assembly of flagella. Red indicates proteins observed to be at significantly greater abundance in −10°C after 8 weeks. Blue indicates proteins observed to be at significantly lower abundance at −10°C after 8 weeks. Black indicates the protein was observed, but had no significant increase or decrease between the cells states. Grey protein complexes with no bold text are indicative of proteins that were not observed in this proteomic profile.

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